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Hepatitis C Genotypes and Subtypes in Saudi Arabia

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Hepatitis C virus (HCV) genotypes are diverse geographically. Infectivity, pathogenicity, and sustained response to treatment may be influenced by HCV genotypes/subtypes. This study examined the relative distribution of hepatitis C genotypes and subtypes among isolates from 84 individuals with chronic active hepatitis (CAH), 39 haemodialysis patients, and 31 intravenous drug addicts, of Saudi Arabian origin. Reverse transcription-polymerase chain reaction (RT-PCR) using specific primers from the 5′-UTR was performed and amplified products were genotyped/subtyped using a commercial reverse phase hybridisation technique (Innolipa HCV 11, Innogenetics, Belgium). Seventy-four percent of the CAH patients were found to be genotype 4 (4c/4d: 33%; 4h: 14%; 4e: 7%; 4: 20%) but other subtypes such as 1b: 14%, 2b: 4%, 3a: 5%, 5a: 1%, and 6a: 1%, were also detected. A history of blood transfusion was disclosed in only 10% of the CAH group. The pattern among haemodialysis patients was as follows: genotype 4: 49% (4h: 13%; 4: 36%); 1a: 33%, 1: 3%; 1b: 10%; and 5a: 5%. The intravenous drug addict group showed 39% subtype 1b, but other subtypes such as 9% for 1a; 3% for 2a; 36% for 4; 3% for 5a; and 9% for 3a were seen. It is concluded that genotype 4 is predominant among our HCV isolates from CAH patients but subtype 1a and 1b have emerged among our haemodialysis and intravenous drug addict cases, respectively. A significant relationship between the viral genotype and the source of infection has emerged among Saudi groups at high risk for hepatitis C virus. ***J. Med. Virol. 58:44–48, 1999.***

KEY WORDS: HCV; genotypes; subtypes; PCR; CAH; CRF; IVDA; Saudi Arabia

INTRODUCTION

Recent advances in basic virology and molecular biology have contributed to our knowledge of the epidemiology, pathogenicity, and response to treatment of hepatitis C virus (HCV) infections. However, issues such as natural history and clinical outcome of HCV infection remain sketchy and unresolved. The possibility that host and viral factors may influence the progression of HCV infection continues to be investigated globally. Of the many parameters such as age at exposure, histological characteristics of the liver, duration of the disease, the abuse of alcohol, and the mode of viral transmission that appear to affect the natural history of the infection, viral genotype and subtypes seem to be the most important predictors of pathogenicity and response to interferon treatment [Pozzato et al., 1991; Kohara et al., 1995]. HCV exhibits a substantial nucleotide variability based on genomic differences. As much as a 30% variation is observed among HCV genotypes and less than 10% are found within subtypes [Simmonds et al., 1993a; Bukh et al., 1994; Stuyver et al., 1994]. Classification based on similarities between genomic sequences have led to the identification of more than 10 major genotypes and as many as 30 subtypes [Bukh et al., 1993a, 1994; Simmonds et al., 1994]. There is a distinct geographical pattern in the distribution of HCV genotypes. Whereas genotypes 1 and 2 are more prevalent in countries in Europe, North America, and Japan [Pawlotsky et al., 1995; Mizoguchi et al., 1996; Zein et al., 1996], others like 3, 4, 5, and 6 are restricted geographically [Bukh et al., 1993b; Dusheiko et al., 1994; Davidson et al., 1995; Tokita et al., 1994, 1996]. The genetic diversity of the virus may explain the variable clinical course of the disease [Kobayashi et al., 1996] and the rather poor sustained response to treatment in certain genotypes [Mahaney et al., 1994; Nousbaum et al., 1995; Pawlotsky et al., 1996].

Hepatitis C genotyping may help to define the high percentage of sporadic infections for which no obvious source can be found [Alter et al., 1989; Gordon et al.,

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1993]. Saudi Arabia, like most Middle Eastern countries, shows an intermediate endemicity for HCV. Seroprevalence rates ranging from 0.9 to 5% have been reported among children and adults respectively [Al-Faleh et al., 1991; Bakir, 1992]. However, certain high risk groups are known to have higher prevalence ranging from 20% in drug addicts, 40% among chronic liver disease patients, and up to 95% among long-term Saudi haemodialysis patients [Huraib et al., 1995; Shobokshi, 1997]. Some studies have indicated the predominance of genotype 4 in this region [Al-Faleh et al., 1995; Mellor et al., 1995; Al-Ahdal et al., 1997], but a high sequence heterogeneity is known to be associated with HCV type 4. Hence HCV subtype distribution is needed to provide clues for studying epidemiology, the mode of transmission, and response to treatment.

The goal of this study was to determine the HCV genotype and subtype distribution among hepatitis high-risk groups in Saudi Arabia.

PATIENTS AND METHODS

Sera from 84 histologically confirmed chronic active hepatitis (CAH) patients from King Abdulaziz University Hospital in Jeddah, western region of Saudi Arabia, were carefully stored at −70°C and later tested for HCV genotypes/subtypes. Thirty-nine consecutive chronic renal failure (CRF) patients who regularly attended (thrice a week) the haemodialysis unit at the Nephrology Centre at Riyadh Medical Complex constituted the CRF group. Sera from 31 intravenous drug addicts (IVDA) with confirmed HCV positivity and HCV-RNA seropositivity, attending Al-Amal Hospital in Riyadh, formed the IVDA group.

Inclusion criteria for samples comprised persistent, elevated alanine transaminase (ALT) of more than twice the upper limit of normal, for 6 months or more, anti-HCV enzyme-linked immunosorbent assay (ELISA) positivity confirmed by Recombinant Immunoblot Assay (Ortho Diagnostics Systems Inc., Raritan, NJ) and polymerase chain reaction (PCR) HCV-RNA positivity (Amplicor Roche Molecular Systems, Basel, Switzerland). Patients with HBsAg positivity, autoimmune disease, alcohol- or drug-induced liver disease, decompensated cirrhosis, hepatic failure, or abnormalities of haematological indices were excluded from the study.

Sample Preparation

Viral RNA was extracted from 100 μl of serum in a complete RNase-free environment, using lysis buffer (68% guanidine thiocyanate, 1 M Tris-HCl pH 7.5, 1% dithiothreitol, and 1 μg glycogen) as per the manufacturer's recommended protocol (Hoffman-La Roche Ltd. Basel, Switzerland).

Complementary DNA (cDNA) Synthesis

RNA was converted to cDNA at 42°C using avian myeloblastosis virus-reverse transcriptase enzyme (AMV-RT, Gibco-BRL, Gaithersburg, MD) as per the manufacturer's instructions.

First Round PCR

Ten microliters of reversed transcribed cDNA were incubated in 50 μl reaction volume containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleotide triphosphate, 10 μl of biotinylated random outer primers from 5′-noncoding region (NCR) (Innolipa) and 0.5 U of Taq polymerase (Amplitaq, Perkin Elmer, Norwalk, CT). Amplification was carried out for 40 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) in a Perkin Elmer Thermocycler.

Positive control (plasmid-cloned HCV cDNA sequences) and negative control (serum from seronegative blood donor) were run simultaneously with the test samples throughout the assay to ensure that no contamination or polymerase inhibition occurred.

Electrophoresis

The amplified product was analysed on 2% agarose gel with a positive PCR yielding a 300-bp fragment. A molecular weight marker (ϕ174/Hae III) was run simultaneously and DNA bands were visualised under ultraviolet (UV) light.

Second Round PCR

Amplified products showing no bands on electrophoresis were next subjected to a second round PCR, using 5 μl amplified products from the first round, 10 μl of biotinylated inner primers from 5′-NCR (Innolipa), and Taq polymerase enzyme in the same conditions as described for the first round PCR. Electrophoresis was repeated as described above. Ten microliters of positive amplified products were used for the Innolipa Genotyping Assay.

Hepatitis C Viral Genotyping

Based on variations found in the highly conserved 5′-NCR genome for the different HCV genotypes and subtypes, specific probes tailed with a poly (T)-tail using terminal deoxynucleotidyl transferase were bound to nitrocellulose membranes solid phase. The following antigens were screened for: 1a; 1b; 1; 2a/2c; 2b; 2k; 3a; 3b; 3c; 3; 4a; 4b; 4c/4d; 4e; 4f; 4h; 4; 5a; 6a; and 10a. The biotinylated amplified products were reverse hybridised to the specific probes at 50°C as per the manufacturer's instructions (Innolipa). This method allowed stringent discrimination at the subtype level.

After hybridisation, streptavidin-labelled alkaline phosphatase was added to bind to the biotinylated hybrid on nitrocellulose membrane. 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) substrate was then added to react specifically with the alkaline phosphatase to produce a purple brown precipitate at the position of a perfect match between the probe and the biotinylated PCR product.

Interpretation

Using a chart provided by the manufacturer, it was possible to deduce the genotype or the subtype based on the precipitation bands on the nitrocellulose cellulose paper.

TABLE I. HCV Genotypes and Subtypes Among CAH, CRF, and IVDA in Saudi Arabia

Type/subtype	CAH (%) (n = 84)	CRF (%) (n = 39)	IVDA (%) (n = 31)[a]
1	—	3	—
1a	—	33	9
1b	14	10	39
2a	—	—	3
2b	4	—	—
3a	5	—	9
4	20	36	21
4c/4d	33	—	12
4e	7	—	—
4h	14	13	3
5a	1	5	3
6a	1	—	—

CAH, chronic active hepatitis; CRF, chronic renal failure; IVDA, intravenous drug addicts.
[a]One patient from IVDA group had mixed infections: 1a, 1b, and 4.

Statistical Analysis

Comparison of data from various high-risk groups were performed using χ^2 and Fisher's exact test whenever appropriate. Statistical significance was defined as $P < .05$.

RESULTS

As shown in Table I, 74% (62 of 84) of CAH patients (sex ratio 4:1, mean age ± SD = 41.2 ± 8.6 years) were found to be genotype 4, ($\chi^2 = 7.45$; $P = .006$). The subtype distribution pattern was as follows: 4c/4d: 33%; 4e: 7%; 4h: 14%; 4: 20%; 1b: 14%; 3a: 5%; 5a: 1%; and 6a: 1%.

Forty-nine percent of CRF patients (sex ratio 1:1, mean age ± SD = 46.6 ± 15.5 years) on regular maintenance haemodialysis were found to be genotype 4. The genotype/subtype distribution was: 36% for type 4 and 13% for subtype 4h; 33% for 1a (Fisher's exact test; $P = .0001$); 10% for 1b; 3% for 1; and 5% for 5a.

Thirty-six percent of IVDA patients (all males, mean age ± SD = 31.0 ± 5.5 years) were found to be genotype 4. The following subtypes were observed: subtype 4c/4d: 12%; type 4: 21%; subtype 1a: 9%; subtype 1b: 42%, ($\chi^2 = 10.17$; $P = .001$); subtype 2a: 3%; subtype 3a: 9%; and subtype 5a: 3%.

DISCUSSION

This study confirms the predominance of genotype 4 in chronic active hepatitis C patients in the western region of Saudi Arabia. Similar studies from central region of Saudi Arabia have reported genotype 4 predominance among patients with chronic liver disease [Al-Faleh et al., 1995; Al-Ahdal et al., 1997]. A high prevalence of subtype 4c/4d in our genotype 4 patients was observed. Reports from neighbouring Gulf countries have also confirmed HCV type 4 predominance for the Middle East region.

The presence of the other major genotypes in our community at much lesser prevalence rates confirms the ubiquity of HCV in this region. Initial reports indicate that genotype 4 is poorly responsive to alpha interferon treatment [El-Zayadi et al., 1996], but more studies will be required to ascertain the pathogenicity and natural history of this genotype. Subtype variation can be expected due to the high genomic heterogeneity within the type 4. The clinical significance of subtype 4c/4d is yet to be determined but its epidemiological significance is eminent. The route of acquisition in chronic HCV patients tends to be ill defined in this community. Only 10% of our chronic active cases had a history of blood transfusion. However, with the natural history of HCV infection dependent on host and viral factors, more work is required to establish the clinical significance of various subtypes of genotype 4. Other genotypes such as 1b and 3a were present but to a lesser degree. Saudi Arabia hosts a large migrant population from countries such as Egypt, where HCV prevalence is among the highest in the world, with genotype 4 as the most predominant [Simmonds et al., 1993b; McOmish et al., 1994; El-Zayadi et al., 1996].

Other groups from the India subcontinent and Far Eastern countries such as Bangladesh, Thailand, and Indonesia where subtype 3a predominate have constituted a major proportion of our labour force [Mori et al., 1992; Okamoto et al., 1993, 1994]. There is no indication of a major crossover of subtype 4a or subtype 3a in our community. This finding is probably because HCV transmission requires close contact, which is rare between Saudis and their migrant workers.

Hepatitis C viral infection is prevalent in haemodialysis patients throughout the world [Zeldis et al., 1990; Chan et al., 1993]. Huraib and co-workers [1995] reported a 94.7% exposure rate among Saudi patients with end-stage renal failure in one study. Several factors such as blood transfusion, duration of maintenance dialysis, partial immunodeficiency, and frequent parenteral manipulations of patients and congestion within haemodialysis units are operative in the transmission of HCV in this high-risk group [Hardy et al., 1992; Knudsen et al., 1993]. Recent improvement of preventive measures including the use of dedicated equipment and staff for positive patients seem to have reduced nosocomial transmission within haemodialysis units [Vagelli et al., 1992; Jadoul et al., 1993; Pereira, 1998]. Subtype 1a has emerged as a significant subtype in our chronic renal patients. It is possible that subtype 1a was introduced into this group due to our heavy dependence in the past on foreign blood supply for transfusion in the 1980s, when a screening test for HCV was unavailable.

Most of the blood was from commercial sources in the US. HCV subtype 1a is common in the US [Zein et al., 1996]. Strict adherence to universal precautions and infection control procedures must be our main objective. Our intravenous drug users showed a high prevalence of subtype 1b, which was significantly different from the major subtypes found among CAH and CRF patients. The fact that only genotypes 1 and 2 were found in nearly 90% of our IVDA cases may contribute to a focal point of infection.

Genotype 3, recently introduced into European intra-

venous addicts [Silini et al., 1995; Love et al., 1996; Shah et al., 1997] was detected in less than 10% of our IVDA group.

Because drug abuse of any form is a serious crime in the Kingdom of Saudi Arabia, the practise of sharing needles is unlikely to be common. Heroin abusers are predominantly men of high socioeconomic status and who travel abroad frequently to indulge in their drug habits. In this setting, it is possible that they might share needles abroad. Subtype 1b is prevalent in North Africa [Benani et al., 1997] and may be a focal point for introduction of this subtype into Saudi drug addicts. History of blood transfusion was not a significant factor among Saudi addicts.

It is concluded that genotype 4 is predominant among Saudi CAH patients, with subtype 4c/4d being the most predominant. History of blood transfusion among CAH patients is rare and the route of most infections is not known. However, there is a significant emergence of subtype 1a among our haemodialysis patients and subtype 1b among Saudi IVDAs, indicating a close relationship between certain HCV subtypes and routes of transmission in high-risk groups. HCV genotyping will continue to play an important epidemiological role on which prevention schemes can be based.

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